

Assessment of the Genotoxic and Immunological Parameters of *Heterobranchus bidorsalis* Exposed to Sub Lethal Concentrations of Dichlorvos

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How to Cite

Osho, F.E., Ajani, E.K., Jenyo-Oni A., Hassan, T., Awoyemi, I.E. (2026). Assessment of the Genotoxic and Immunological Parameters of *Heterobranchus bidorsalis* exposed to Sub Lethal Concentrations of Dichlorvos. *Aquaculture Studies*, 26(4), AQUAST2843. <http://doi.org/10.4194/AQUAST2843>

Article History

Received 10 August 2025

Accepted 02 April 2026

First Online 13 May 2026

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Keywords

Xenobiotics

Micronuclei

Oxidative Stress

Fish Erythrocyte

Abstract

Dichlorvos, an organophosphate compound commonly used as pesticide, contains xenobiotics capable of damaging aquatic ecosystems. However, there is inadequate information on its effects in aquatic vertebrates such as *Heterobranchus bidorsalis*. This study investigated its 96h LC₅₀ toxicity, alterations in immunological, oxidative parameters and cytogenotoxicity in *H. bidorsalis*. Juvenile *H. bidorsalis* (n=250) were exposed to 0.00, 1.0, 2.2, 4.8 and 10 mg/l of Dichlorvos and later to sub-lethal concentrations corresponding to 1/40, 1/20 and 1/10 of the 96-h LC₅₀ (1.16 mg/l) for 21 days. Exposure induced concentration and time-dependent suppression of immune functions in *H. bidorsalis*. Total immunoglobulin and serum lysozyme levels dropped significantly at 0.12 mg l⁻¹ (0.004 g dl⁻¹ and 0.002 g dl⁻¹, respectively). Oxidative stress increased significantly, with lipid peroxidation rising from 0.001 to 0.011 μmol mg⁻¹ protein (P<0.05). Frequencies of MN elicited 2.2 folds increase at the highest exposure level compared to the negative control in dose and time dependent pattern (P<0.05) after 21 days. Similarly, enucleated, bi-nucleated and budded nuclei erythrocytes significantly (P<0.05) increased in 2.78, 23.6 and 10.4 folds, respectively. Results indicate that Dichlorvos weakens immune defense, disrupts oxidative balance, and induces cellular damage in fish.

Introduction

Several threats, mainly from anthropogenic activities are currently challenging the integrity and stability of the aquatic ecosystems globally (Ajani and Osho, 2019). Among these, agrochemicals, particularly pesticides, are sources of toxic compounds with profound impacts on diversity of aquatic environments which are the final sink. Dichlorvos (2, 2-dichlorovinyl dimethyl phosphate), an organophosphate pesticide is widely used in agricultural fields, farm animals, stored grains and ectoparasitic infections (Rao et al., 2017). However, its indiscriminate use and discharge into water bodies have made its use of particular concern, raising

serious health and ecological concern (Das, 2013). In addition, runoff from adjacent agricultural lands inevitably contaminates water bodies, negatively impacting wild aquatic life (Liang et al., 2025)

Aquatic organisms are often the final recipients of these contaminants (Firat et al., 2011). Fishes are exposed to pesticides through direct absorption via the skin, feeding and uptake by gills during respiration (Bhat et al., 2010). Dichlorvos residues may also occur in bottom sediments, increasing exposure risk for benthic and bottom-dwelling fish. Stress induced by exposure to Dichlorvos has been linked with DNA damage in living cells and initiation of adverse effects at the cellular, individual, community and population levels (Lee et al.,

2013). Because fish exhibit a slower rate of DNA repair relative to mammals, they serve as a sensitive indicator species for evaluating environmental genotoxicity (Espina and Weis, 1995; Osho et al., 2023). The use of micronucleus assay as genetic biomarker is one of the most applicable techniques to identify genomic alterations in erythrocytes of fish due to its simplicity in assessment of nuclear abnormalities (Bolognesi and Hayashi, 2011). When combined with immunological and oxidative stress biomarkers, it offers a comprehensive framework for assessing sub-lethal pesticide toxicity and early biological effects.

Catfishes, particularly those in the *Clarias* and *Heterobranchus* genera are the second most cultured fish group on the African continent. They are also commonly consumed fish groups in the region, contributing to food security and economic prosperity of the region (Osho et al 2016; FAO, 2024). Their benthopelagic nature makes them highly vulnerable to Dichlorvos. At the same time, the use of organophosphate pesticides, including Dichlorvos, remains widespread in developing countries such as Nigeria for agricultural, veterinary and domestic pest control, often under conditions of weak regulation and inappropriate application practices. While several authors have documented systemic toxicity and genomic effects of Dichlorvos contamination on various commercially important fish species (Amaeze et al., 2020; Anukwu et al., 2020; Trivedi et al., 2021), there is dearth of information on the effect of Dichlorvos on *H. bidorsalis*, an important food fish in Africa. Therefore, the objective of this study was to assess the genotoxic, immunological and oxidative stress responses of *H. bidorsalis* exposed to sub-lethal concentrations of Dichlorvos.

Materials and Methods

Test Chemicals

Commercial DD Force™, with active ingredient (Dichlorvos 2, 2-dichlorovinyl dimethyl phosphate) 1000 EC and Cyclophosphamide in analytical grades were used.

Test Fish

Heterobranchus bidorsalis juveniles (10.2±0.1 g; 13.3±0.7 cm) obtained from a reputable fish farm in Ibadan were used for this study. They were acclimatised under laboratory conditions (ambient temperature 28°C±0.5; Light: Darkness, 12: 12 h) for fourteen days in rectangular glass tanks, three-quarters filled with dechlorinated water. The fish were fed with 2mm commercial fish pellet at 5% body weight twice daily and the stock water was refreshed every other day. The acclimatisation tank was continuously aerated with 220 V air pumps throughout the period. Feeding was

stopped 24 hours before commencement of the experiments.

Experimental Procedure and Acute Toxicity Test

A preliminary range-finding assay (0.1–100 mg/L) was conducted following Odiete (1999). Based on the outcomes of the range-finding test, four concentrations of Dichlorvos (1.0, 2.2, 4.8 and 10 mg/L) and a control (dechlorinated tap water; 0.00 mg/L) were selected for the 96-h acute toxicity test (static bioassay). Randomly selected *H. bidorsalis* juveniles were assigned to each concentration with 10 fish per treatment in triplicate tanks. Stocking density complied with space-factor recommendations by Reish and Oshida (1987). Fish were not fed during the acute toxicity study. Water in the tank was refilled daily with freshly prepared concentrations. Mortality and clinical signs of toxicity were monitored by visual observation at 3-h intervals, and dead fish were removed immediately. Water quality parameters (temperature, pH and dissolved oxygen) were measured at 0900hours every morning, following standard procedures and averages were analysed over the culture period. The LC₅₀ concentration was determined after the 96 hours exposure as a probit analysis using the probit mortality versus the logarithm concentration according to Finney (1971).

Sub-lethal Toxicity

Based on the derived 96-h LC₅₀ value, three sub-lethal concentrations corresponding to 1/40, 1/20 and 1/10 of the 96-h LC₅₀ (1.16 mg/l) were selected for chronic exposure resulting to 0.03, 0.06 and 0.12, respectively. Fifteen juveniles were exposed to each sub-lethal concentration in a semi-static bioassay system. Dechlorinated tap water (DTW) served as the negative control, while cyclophosphamide (4 mg/L) 4 mg/L was included as a positive genotoxic control. Test solutions were renewed every 48 h to maintain chemical stability and adequate dissolved oxygen levels. Fish were fed twice daily at 5% body weight. On 1, 7, 14 and 21 days of the exposure, peripheral blood was collected from test fish, per sampling time, from each of the treatment and control groups via caudal vein into EDTA bottles (Osho et al., 2016) for micronucleus and nuclear abnormalities.

Immunological and Oxidative Stress Analyses

Total immunoglobulin, serum lysozyme activity, serum albumin and white blood, oxidative stress indices were used for immunological assay (Svobodava et al., 2001; Pitaksong et al., 2013). Oxidative stress indices evaluated were total protein (TP), malondialdehyde (MDA) and nitric oxide (NO), glutathione (GSH), glutathione peroxidase (GPx) and superoxide dismutase (SOD) (Habig et al., 1974; Olaleye et al., 2007). These were analysed days 1 and 21 of exposure, respectively.

Micronucleus Analysis

Peripheral blood samples were collected from fifteen fish in the test and control groups at 1, 7, 14 and 21 days of exposure. For each fish, four pre-cleaned and grease free microscope slides were prepared by making thin blood smears. The prepared slides were air-dried for 24-h, fixed in absolute (98%, v/v) cold methanol (4°C) for 30 min and counter stained with 5% Giemsa and 10% May-Grunwald stains for 20 min. Three thousand erythrocytes per fish were scored for micronucleus (MN) induction at $\times 1000$ magnification (oil immersion) in accordance with standard protocols (Alimba et al., 2019). Nuclear abnormalities (NAs) were also scored as cytotoxic parameters at the same magnification to determine the frequency of bi-nucleated cells, enucleated and budded nuclei. The slides were coded randomly, and scoring was performed blind to treatment group to minimise bias.

Statistical Analysis

Data obtained from the 96h mortality were analysed using probit method (Finney, 1971) with SPSS™ version 23.0® computer program. The analysed acute toxicity indices were presented as LC_5 , LC_{50} and LC_{95} . Data for physico-chemical, immunological, oxidative stress, micronucleus and nuclear abnormalities data were presented as mean \pm standard error (SE). The differences among various groups according to exposure duration was determined using One-way ANOVA, while Dunnett multiple post hoc test was used to compare the level of significance ($P < 0.05$) of each treated group with the negative control using Graphpad prism 8.0® computer program.

Results

Water Quality Assessment

The physico-chemical parameters of water recorded during both the 96-h acute and 21-day sub-lethal exposures of *H. bidorsalis* to Dichlorvos are presented in Tables 1 and 2. During the 96-h acute exposure, pH decreased significantly with increasing Dichlorvos concentration, shifting from neutral in the control (7.12) to slightly acidic at the highest concentration (5.70 mg/L). A similar trend was observed during the 21-day sub-lethal exposure, where pH declined progressively with increasing pesticide concentration, with a significant reduction at 0.13 mg/L. Similarly, DO concentration also declined with increasing Dichlorvos concentration, from 5.40 mg/L in the control to 3.90 mg/L at the highest concentration in the acute exposure and 5.80 mg/L to 4.95 mg/L. Temperature, however increased with increasing Dichlorvos concentration in both acute and sub-lethal exposures. In the acute test, temperature rose from 24.40°C in the control to 26.00°C at 10.0 mg/L, while in the sub-lethal test, temperature increased from 24.70°C in the control to 27.60°C at 10.0 mg/L. The values varied significantly ($P < 0.05$) from the control, although the values obtained are still within the acceptable range for water quality in aquaculture.

Acute Toxicity Response

The acute toxicity of Dichlorvos to *H. bidorsalis* juveniles over a 96-hour exposure period is summarised in Tables 3 and 4. No mortality was observed in the control group throughout the exposure period or within the first 24 h. Mortality was first recorded at 48 h at

Table 1. Physico-chemical parameters of water during the 96-hour exposure of *H. bidorsalis* to varying concentrations of Dichlorvos

Concentration (mg/l)	pH	Temperature (°C)	Dissolved Oxygen (mg/l)
0.00 (DTW)	7.12 \pm 0.02	24.40 \pm 0.14	5.40 \pm 0.04
1.00	6.92 \pm 0.15	24.90 \pm 0.12	4.72 \pm 0.04*
2.20	6.30 \pm 0.44*	25.00 \pm 0.10*	4.50 \pm 0.38*
4.80	6.00 \pm 0.10*	25.90 \pm 0.15*	4.3 \pm 0.34*
10.00	5.70 \pm 0.03*	26.00 \pm 0.03*	3.90 \pm 0.16*

Values are expressed mean \pm standard error (SE, n = 10). Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett multiple post hoc tests. Values marked with Asterisks (*) differ significantly from the negative control ($P < 0.05$). DTW: Dechlorinated tap water (Negative control).

Table 2. Physico-chemical parameters of water during the 21-day sub-lethal exposure of *H. bidorsalis* to varying concentrations of Dichlorvos

Concentration (mg/l)	pH	Temperature (°C)	Dissolved Oxygen (mg/l)
0.00 (DTW)	7.09 \pm 0.13	24.70 \pm 0.04	5.80 \pm 0.03
CYP	5.30 \pm 0.01*	27.60 \pm 0.01*	3.50 \pm 0.06*
0.03	6.91 \pm 0.00	24.74 \pm 0.02	5.50 \pm 0.11
0.06	6.90 \pm 0.02	24.81 \pm 0.01	5.00 \pm 0.08*
0.13	6.80 \pm 0.15*	24.90 \pm 0.13*	4.95 \pm 0.14*

Values are expressed mean \pm standard error (SE, n = 15). Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett multiple post hoc tests. Values marked with Asterisks (*) differ significantly from the negative control ($P < 0.05$). DTW: Dechlorinated tap water (Negative control); CYP: Cyclophosphamide (Positive control).

concentrations of 4.8 and 10 mg/L and increased with both concentration and exposure duration, indicating a clear dose- and time-dependent response. Probit values of 4.82 and 5.84 were obtained at concentrations of 1.00 and 2.20 mg/L, respectively.

Estimated lethal concentrations (LC₅, LC₅₀ and LC₉₅) declined progressively with exposure time (Table 4). The LC₅₀ decreased from 11.17 mg/L at 48 h to 2.67 mg/L at 72 h and 1.16 mg/L at 96. Similar trends were observed for LC₅ and LC₉₅ values. The probit regression slope increased from 1.52±0.31 at 48 h to 2.97±0.38 at 96 h, indicating a steeper concentration–response relationship at longer exposure durations.

Immunological and Oxidative Stress Responses

After 21 days of exposure (Table 5), reductions in all immunological indices were more pronounced than those observed at day 1. Immunoglobulin concentration, lysozyme activity, albumin level and white blood cell count decreased significantly in all Dichlorvos-treated groups relative to the control (P<0.05), with the greatest reductions recorded at 0.12 mg/L.

Changes in immunological parameters of *H. bidorsalis* following exposure to Dichlorvos are presented in Tables 5 and 6. At day 1 of exposure (Table 5). All treatments exposed to Dichlorvos and

Table 3. 96-hours concentration-response of *H. bidorsalis* to Dichlorvos exposure

Conc. (mg/l)	Conc. (Log ₁₀)	No of fish exposed	Number of deaths				Total mortality	% mortality	Probit
			24h	48h	72h	96h			
0.00(DTW)	-	10	0	0	0	0	0	0	
1.00	0.00	10	0	0	2	2	4	40	4.82
2.20	0.34	10	0	0	4	4	8	80	5.87
4.80	0.68	10	0	2	5	3	10	100	
10.00	1.00	10	0	4	5	1	10	100	

Number of fish (n)= 10, Concentrations (mg/L) are log₁₀-transformed and analysed using probit analysis. Dashes (–) indicate mortality outside the probit range. DTW: Dechlorinated tap water (Negative control).

Table 4. 96-hour LC₅₀ acute toxicity of Dichlorvos on *H. bidorsalis* at 95% confidence limit)

Time	LC ₅	LC ₅₀	LC ₉₅	Slope±SE	Probit line equation
48h	2.87 (0.02–4.81)	11.17 (6.99–407.61)	43.47 (16.77–500.77)	1.52±0.31	Y= -1.78+1.52X
72h	0.45 (0.0–1.00)	2.67 (1.40–4.56)	15.85 (7.64–201.35)	2.14±0.25	Y= -0.90+2.14X
96h	0.41 (0.01–0.76)	1.16 (0.42–1.69)	3.33 (2.14–31.95)	2.97±0.38	Y= 0.81+2.97X

Number of fish (n= 15) Lethal concentration values (mg/L) with 95% confidence limits and probit regression parameters were obtained using probit analysis of mortality data. Slope values are presented as slope±standard error (SE). LC₅, LC₅₀ and LC₉₅ represent concentrations causing 5%, 50% and 95% mortality, respectively.

Table 5. Changes in Immunological parameters of *H. bidorsalis*, exposed to Dichlorvos toxicity at 21 days of exposure

Conc. (mg/l)	Immunological parameters			
	Immunoglobulin (g/dl)	Lysozyme (g/dl)	Albumin (g/dl)	White blood cell (x10 ³ L)
0.00 (DTW)	0.012±0.00	0.005±0.00	3.313±0.16	4.600±0.10
CYP	0.003±0.00*	0.001±0.00*	1.000±0.00*	2.010±0.00*
0.03	0.008±0.00*	0.004±0.00*	2.237±0.12*	3.257±0.60*
0.06	0.006±0.00*	0.003±0.00*	1.887±0.04*	2.843±0.13*
0.12	0.004±0.00*	0.002±0.00*	1.368±0.12*	2.130±0.03*

Values are expressed as mean±standard error (SE, n= 15). Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett multiple post hoc tests. Values marked with Asterisks (*) differ significantly from the negative control (P<0.05). DTW: Dechlorinated tap water (Negative control); CYP: Cyclophosphamide (positive control).

Table 6. Changes in immunological parameters of *H. bidorsalis*, exposed to Dichlorvos toxicity at day 1 of exposure

Conc. (mg/l)	Immunological parameters			
	Immunoglobulin (g/dl)	Lysozyme (g/dl)	Albumin(g/dl)	White blood cell (x10 ³ L)
0.00 (DTW)	0.025±0.00	0.006±0.00	3.507±0.14	5.687±0.04
CYP	0.001±0.00*	0.001±0.00*	2.000±0.00*	4.033±0.06*
0.03	0.08±0.01*	0.005±0.00*	2.600±0.10*	5.590±0.02*
0.06	0.006±0.00*	0.003±0.00*	2.467±0.21*	5.200±0.10*
0.12	0.004±0.00*	0.002±0.00*	2.133±0.06*	4.473±0.20*

Values are expressed as mean±standard error (SE, n= 15). Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett multiple post hoc tests. Values marked with Asterisks (*) differ significantly from the negative control (P<0.05). DTW: Dechlorinated tap water (Negative control); CYP: Cyclophosphamide (positive control).

cyclophosphamide (positive control) showed substantial ($P<0.05$) decreases in total immunoglobulin, serum lysozyme activity, serum albumin and white blood cells levels, compared with the negative control (Table 5). After 21 days of exposure (Table 6), the same pattern of immunological reduction was observed, with a more pronounced change across all measured parameters.

Oxidative Stress Responses of *H. bidorsalis* Exposed to Dichlorvos

Oxidative stress responses of *H. bidorsalis* following exposure to Dichlorvos are presented in Tables 7 and 8. At day 1 of exposure (Table 7), all measured oxidative stress indices differed significantly from the control ($P<0.05$). Total protein levels were reduced in exposed groups relative to the control. Activities of antioxidant defence enzymes such as GSH, GPx and SOD were also significantly lower in Dichlorvos-treated fish, as were nitric oxide and malondialdehyde levels. Except for total protein, all parameters showed a decline relative with increasing concentration. After 21 days of exposure (Table 8), the same pattern of response was observed across all measured parameters.

Genotoxic Effects of Dichlorvos Exposure to *H. bidorsalis*

Nuclei abnormalities observed in the peripheral erythrocytes of *H. bidorsalis* intoxicated with Dichlorvos were micronuclei (MN), binucleated cells (BN), budded nuclei and enucleated cells (Figure 1). There was

concentration and time dependent increase in frequencies in micronuclei induction in peripheral erythrocytes of test fish exposed to Dichlorvos as shown in Figure 2. At day 1 there were minimal MN frequencies of 0.02% at the highest concentration (0.12 mg/L) while at day 7 of exposure, the highest concentration treatment induced MN frequencies of approximately 0.43%, representing a 1.8 fold increase over the negative control (0.25%), demonstrating rapid onset of chromosomal damage. After 14 days, MN frequencies ranged from 0.32% in negative control to 0.62% in the 0.12 mg/L treatment yielding an approximate 1.7 fold difference. The most pronounced effects emerged after 21 days of chronic exposure, where the highest concentration reached 0.833% compared to the negative control's 0.387%, representing a 2.2 fold increase ($P<0.05$).

On day 1, the frequencies of enucleated erythrocytes were minimal, with the highest concentration (0.12 mg/L) showing 0.040 ± 0.01 . By day 7, frequencies had increased substantially, with 0.12 mg/L reaching 0.427 ± 0.04 compared to 0.153 ± 0.02 in the negative control, indicating a rapid onset of chromosomal damage. After 14 days, the recorded level ranged from 0.293 ± 0.04 in the negative control to 0.947 ± 0.04 in the 0.12 mg/L treatment, representing a pronounced concentration-dependent effect. The most significant impact was observed at day 21, where chronic exposure to 0.12 mg/L produced 0.833 ± 0.03 compared to 0.387 ± 0.05 in the negative control, demonstrating clear and sustained chromosomal damage ($P<0.05$).

Table 7. Oxidative stress response of *H. bidorsalis*, exposed to Dichlorvos toxicity at day 1 of exposure

Parameters	DTW	CYP	Concentrations (mg/l)		
			0.03	0.06	0.12
TP ($\mu\text{g/dl}$)	4.027 ± 0.07	$2.733\pm 0.12^*$	$3.773\pm 0.05^*$	$3.647\pm 0.05^*$	$3.321\pm 0.05^*$
MDA ($\mu\text{mol/mg protein}$)	0.004 ± 0.03	$0.005\pm 0.00^*$	$0.006\pm 0.00^*$	$0.010\pm 0.00^*$	$0.016\pm 0.00^*$
NO ($\mu\text{mol/mg protein}$)	0.001 ± 0.00	0.010 ± 0.00	0.005 ± 0.001	$0.006\pm 0.02^*$	$0.009\pm 0.00^*$
GSH ($\mu\text{g/ml}$)	0.003 ± 0.01	$0.005\pm 0.00^*$	$0.007\pm 0.00^*$	$0.010\pm 0.00^*$	$0.013\pm 0.00^*$
GPx (units/mg protein)	0.003 ± 0.01	$0.005\pm 0.00^*$	$0.004\pm 0.00^*$	$0.013\pm 0.00^*$	$0.016\pm 0.00^*$
SOD (units/mg protein)	0.002 ± 0.00	$0.005\pm 0.00^*$	$0.010\pm 0.00^*$	$0.013\pm 0.00^*$	$0.016\pm 0.00^*$

TP: Total Protein, MDA: Malonaldehyde, NO: Nitric oxide, GSH: Glutathione, GPx: Glutathione peroxidase, SOD: superoxide dismutase, DTW: Dechlorinated tap water (Negative control); CYP: Cyclophosphamide (positive control). Values are expressed as mean \pm standard error (SE, $n=15$). Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett multiple post hoc tests. Values marked with Asterisks (*) differ significantly from the negative control ($P<0.05$).

Table 8. Oxidative stress response of *H. bidorsalis* exposed to Dichlorvos toxicity at 21 days of exposure

Parameters	DTW	CYP	Concentrations (mg/l)		
			0.03	0.06	0.12
TP ($\mu\text{g/dl}$)	4.012 ± 0.00	$1.783\pm 0.029^*$	$3.390\pm 0.07^*$	$2.833\pm 0.06^*$	$2.500\pm 0.10^*$
MDA ($\mu\text{mol/mg protein}$)	0.001 ± 0.001	$0.002\pm 0.00^*$	$0.003\pm 0.00^*$	$0.005\pm 0.00^*$	0.011 ± 0.00
NO ($\mu\text{mol/mg protein}$)	0.001 ± 0.000	$0.001\pm 0.00^*$	$0.003\pm 0.00^*$	$0.005\pm 0.00^*$	0.007 ± 0.00
GSH ($\mu\text{g/ml}$)	0.012 ± 0.00	$0.002\pm 0.00^*$	$0.004\pm 0.00^*$	$0.007\pm 0.00^*$	0.010 ± 0.00
GPx (units/mg protein)	0.012 ± 0.000	0.010 ± 0.00	$0.002\pm 0.00^*$	$0.005\pm 0.00^*$	0.011 ± 0.00
SOD (units/mg protein)	0.015 ± 0.001	$0.005\pm 0.00^*$	$0.008\pm 0.00^*$	0.010 ± 0.00	0.011 ± 0.00

TP: Total Protein, MDA: Malonaldehyde, NO: Nitric oxide, GSH: Glutathione, GPx: Glutathione peroxidase, SOD: superoxide dismutase, DTW: Dechlorinated tap water (Negative control); CYP: Cyclophosphamide (positive control). Values are expressed as mean \pm standard error (SE, $n=15$). Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett multiple post hoc tests. Values marked with Asterisks (*) differ significantly from the negative control ($P<0.05$).

The occurrence of binucleated erythrocytes (Table 9) increased with both concentration and exposure time, following the pattern: $0.00 < 0.03 < 0.06 < 0.12$ mg/l < positive control. Across all treatment groups, the frequencies of BN erythrocytes were significantly higher ($P < 0.05$) than the negative control after 7, 14, and 21 days of exposure. Specifically, at 7 days, the mean \pm SE values were 0.150 ± 0.04

(negative control), 0.227 ± 0.03 (0.03 mg/l), 0.153 ± 0.03 (0.06 mg/l), 0.560 ± 0.08 (0.12 mg/l), and 0.393 ± 0.06 for cyclophosphamide. This trend continued at 14 and 21 days, with the highest frequencies observed at 0.12 mg/l and the positive control. Enucleated erythrocytes were the most frequently observed of all the abnormalities and it depicted a concentration-time dependent significant ($P < 0.05$) increase as shown in Table 10.

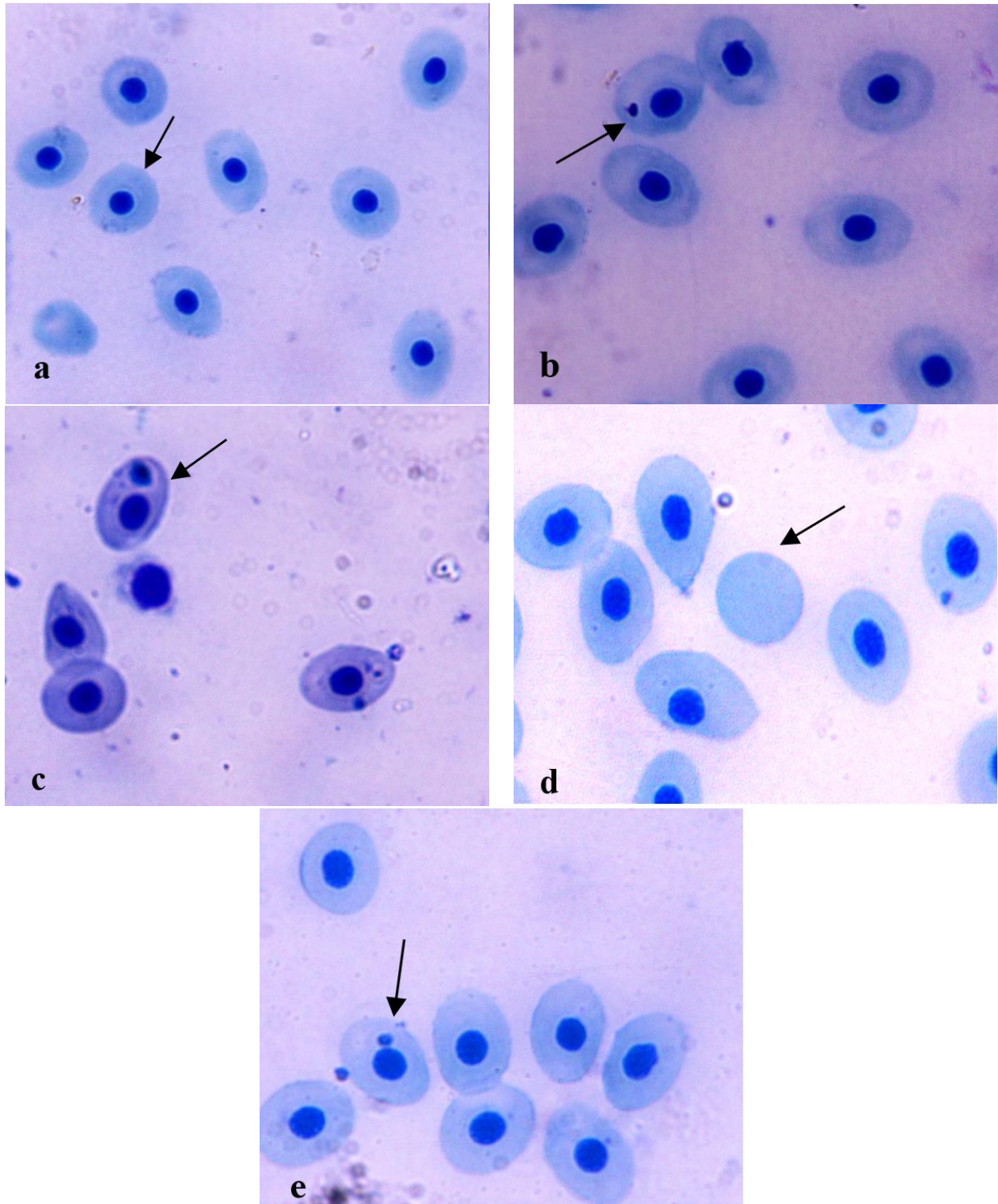


Figure 1. Abnormal nuclei erythrocyte formed in *H. bidorsalis* exposed to Dichlorvos. (a) Normal erythrocyte; (b) MN erythrocyte; (c) binucleated erythrocyte; (d) enucleated erythrocyte; (e) erythrocyte with nuclear bud.

The budded erythrocyte abnormality was the least frequently observed across all treatments. As shown in Table 11, significant increases were only recorded after 21 days of exposure, with frequencies increasing from 0.007 ± 0.01 in dechlorinated tap water to 0.027 ± 0.01 , 0.080 ± 0.02 , and 0.073 ± 0.02 at 0.03, 0.06, and 0.12 mg/L, respectively.

Discussion

From this study, the pH, temperature and dissolved oxygen of the test water were affected by Dichlorvos although the parameters were still within the optimal range for tropical fish (Omitoyin, 2007). The 96h LC₅₀ value of 1.16 mg/l of Dichlorvos to *H. bidorsalis* recorded in this study is close to the LC₅₀ value of 1.32mg/L recorded by Ekpo and Okorie, (2004) on *H. longifilis* but higher than 0.88mg/l and 0.93mg/l reported by Amaeze

et al. (2020) on *C. gariepinus*. The observed variations in toxicity level of Dichlorvos to different fish species may be due to age, weight and length, sex and physiological status as well as water quality (Li et al., 2013). There was progressive stress and uncoordinated swimming behaviour in the test fish with time before death and mortalities increased with increasing concentrations. A similar pattern was reported by Amaeze et al. (2020) when *C. gariepinus* juvenile were exposed to Dichlorvos. Stimulatory effects of the pesticide that inhibit cholinesterase enzymes to acetylcholine receptors in the nervous system of exposed fish can lead to excitation and restlessness.

The present study showed a significant decrease in total immunoglobulin, serum lysozyme and albumin as well as white blood cells in fish exposed to Dichlorvos from day 1 and to 21 days of exposure. The innate immune system is a fundamental defence system in fish.

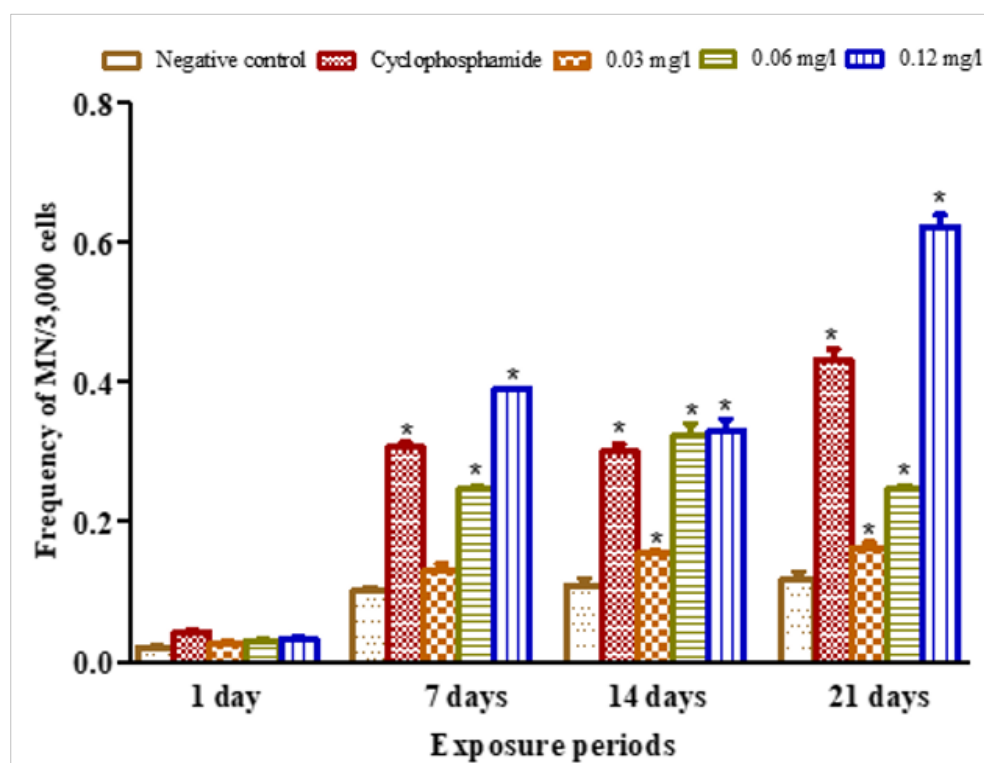


Figure 2. Micronucleated peripheral erythrocytes of *H. bidorsalis* exposed to Dichlorvos. *Significantly different ($P < 0.05$).

Table 9. Frequencies of bi-nucleated erythrocytes in *H. bidorsalis* after exposure to concentrations of Dichlorvos at different exposure periods

Concentrations (mg/l)	Bi-nucleated erythrocytes (Mean \pm SE/3,000 cell)			
	1 day	7 days	14 days	21 days
0.00 (DTW)	0.010 \pm 0.01	0.150 \pm 0.04	0.020 \pm 0.03	0.035 \pm 0.05
(CYP)	0.020 \pm 0.01	0.393 \pm 0.06*	0.447 \pm 0.06*	0.400 \pm 0.02*
0.03	0.010 \pm 0.00	0.227 \pm 0.03*	0.107 \pm 0.07*	0.180 \pm 0.01*
0.06	0.013 \pm 0.01	0.153 \pm 0.03*	0.153 \pm 0.03*	0.207 \pm 0.02*
0.12	0.016 \pm 0.02	0.560 \pm 0.08*	0.660 \pm 0.04*	0.780 \pm 0.03*

Values are expressed as mean \pm standard error (SE, n = 15). Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett multiple post hoc tests. Values marked with Asterisks (*) differ significantly from the negative control ($P < 0.05$).

DTW: Dechlorinated tap water (Negative control); CYP: Cyclophosphamide (positive control).

The total immunoglobulin is an important indicator of the immune status in fish, and this can be by exposure to pesticide (Díaz-Resendiz et al., 2015). The lowered immunoglobulin may be attributed to stress induced by Dichlorvos on the test fish. This result is in tandem with reports of Díaz-Resendiz and Girón-Pérez (2014) that chlorpyrifos diminished the levels of immunoglobulin M in the plasma of *Oreochromis niloticus*. Lysozymes are bacteriolytic enzymes with precise hydrolytic activity, which act against peptidoglycan of bacterial cell walls. Decrease in serum lysosome can be occasioned by suppressing effects of the pesticide on non-specific immune response, particularly production and differentiation of leukocytes (Alishahi et al., 2016). Studies showed similar trend of reduction in lysozyme activity in the plasma of *Oncorhynchus mykiss* and *Cyprinus carpio* exposed to diazinon and phosalone, respectively (Ahmadi et al., 2014; Kaya et al., 2014). Also, it has been reported that the pesticide, diazinon led to a decreased in lysozyme activity in *Barbus sharpeyi* (Alishahi et al., 2016).

Decline in serum albumin indices in experimental groups could be an indicator of stress induced by the pesticide. Serum albumins are used to monitor the course of diseases in immune disorders (Blahova et al., 2016). White blood cells are involved in the regulation of immunological function, and their alterations can relate to immunotoxic potential of substances (Díaz-Resendiz et al., 2015). Decrease in White blood cell was also reported by a Velisek et al. (2008) after rainbow trout were exposed to metribuzin and Blahova et al. (2016) when they exposed Common Carp to Atrazine.

Toxicity of organophosphates is mainly due to the inhibition of acetylcholine esterase. Consequently, cholinergic hyperactivity leads the accumulation of free radicals that causes to lipid peroxidation, a precursor to oxidative stress (Nurulain et al., 2013). Like other organophosphates, it inhibits acetylcholinesterase and the central and peripheral nervous systems (Nurulain et al., 2013). As a result, external and internal changes occur, most of which lead to numerous deformities. Internal changes are mostly at biochemical and genomic levels. It has the potential to induce immunological and oxidative stress whose product peroxynitrite, may react with various amino acid residues in proteins Franco et al. (2010). Excess production of reactive oxygen species can cause oxidative modification of proteins, DNA and lipids, often leading to accelerated cell death (Abello et al., 2009). In the present study, Dichlorvos induced elevated levels of oxidative stress enzymes in *H. bidorsalis*. On the other hand, decrease in blood protein was in tandem with observations documented on fishes exposed to different pesticides such as chlorpyrifos, lambda-Cyhalothrin, cypermethrin buctril (Naqvi et al., 2017). Decreased protein content might also be due to increased proteolytic activity, disturbance of cellular fraction and consequent impairment in protein synthetic machinery (Naqvi et al., 2017). Malonaldehyde is a product of lipoperoxidation and Nitric oxide used as biomarkers of oxidative stress and cellular damage (Ayala et al., 2014). Increased activities of the Malonaldehyde, and Nitric oxide during the exposure time indicated an elevation in the rate of reactive oxygen species production during exposure. According

Table 10. Frequencies of enucleated erythrocytes in *H. bidorsalis* after exposure to concentrations of Dichlorvos at different exposure periods

Concentrations (mg/l)	Enucleated erythrocytes (Mean±SE/3,000 cell)			
	1 day	7 days	14 days	21 days
0.00 (DTW)	0.013±0.01	0.153±0.02	0.293±0.04	0.387±0.05
CYP	0.067±0.02*	0.200±0.02	0.367±0.02	0.807±0.05*
0.03	0.020±0.01	0.180±0.03	0.193±0.02	0.300±0.02
0.06	0.027±0.01	0.280±0.03*	0.420±0.04	0.413±0.03
0.12	0.040±0.01	0.427±0.04*	0.947±0.04*	0.833±0.03*

Values are expressed as mean±standard error (SE, n= 15). Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett multiple post hoc tests. Values marked with Asterisks (*) differ significantly from the negative control (P<0.05). DTW: Dechlorinated tap water (Negative control); CYP: Cyclophosphamide (positive control).

Table 11. Frequencies of budded nuclei erythrocytes in *H. bidorsalis* after exposure to concentrations of Dichlorvos at different exposure periods

Concentrations (mg/l)	Budded nuclei erythrocytes (Mean±SE/3,000 cell)			
	1 day	7 days	14 days	21 days
0.00	0.000±0.00	0.00±0.000	0.007±0.01	0.007±0.01
CYP	0.033±0.01*	0.020±0.02	0.020±0.01	0.033±0.01
0.03	0.000±0.00	0.000±0.00	0.000±0.00	0.027±0.01
0.06	0.000±0.00	0.000±0.00	0.000±0.00	0.080±0.02
0.12	0.000±0.00	0.033±0.02	0.013±0.01	0.073±0.02

Values are expressed as mean±standard error (SE, n= 15). Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett multiple post hoc tests. Values marked with Asterisks (*) differ significantly from the negative control (P<0.05). DTW: Dechlorinated tap water (Negative control); CYP: Cyclophosphamide (positive control).

to Li et al. (2013) oxidative substances in cells may cause an elevation of antioxidant enzymes as a defence mechanism. This is evident in the increased activities of antioxidant enzymes; Glutathione, Glutathione peroxidase and Superoxide dismutase in a bid to protect the cells from oxidative damage (Ighodaro and Akinloye, 2018).

The results of the micronucleus assay showed alterations in cell morphology and presence of nuclear abnormalities as binucleated, budded nuclei, and enucleated cells in peripheral blood cells of *H. bidorsalis* exposed to sub-lethal concentrations of Dichlorvos. The concentration-time dependent increase in frequencies of MN and NA in peripheral erythrocytes of test fish suggests the varying levels of clastogenic and/or aneugenic activities of the test pesticide. Micronucleus has been reported in different fish organs (Srivastava et al., 2016) and their inductions in fishes have been associated with environmental factors as well as rate of cell proliferation (Arkhipchuk and Garanko, 2005).

This pesticide possibly interfered with DNA repair systems (Hartwig, 1998), induced DNA strand breaks and/or mitotic spindle dysfunction (Seoane and Dulout, 2001), and generated reactive oxygen species and glutathione depletion (O'Brien and Salacinski, 1998) in the haematopoietic system of the test fish resulting in the observed cytogenotoxic biomarkers. Significant increase in the frequency of MN in the treated fish suggests that the pesticide induced perturbation in the haematopoiesis of the treated fish which resulted in acentric chromosome fragmentation, acentric chromatid fragmentation or whole chromosome loss that were not included in the daughter nuclei after telophase during cell division (Mateuca et al., 2006). It is also possible that the pesticide induced perturbation in the haematopoiesis of the treated fish which increased gene amplification and were localized to the periphery of the nucleus during S phase of the cell cycle (Shimizu et al., 1998). This may account for the presence of nuclear bud recorded in the exposed test fish.

The various abnormal nucleated erythrocytes were scored to complement MN frequency in the genotoxicity assessment considering that they were significantly found in the Dichlorvos exposed fish compared to the negative control. Increase in the frequency of binucleated erythrocytes suggested blockage of cytokinesis of the dividing cells during erythropoietic process. Increase in enucleated erythrocytes in the pesticide exposed test fish showed that the pesticide is capable of inducing cytotoxic effects in fish via interference with signal transduction pathways which resulted in cell lysis, cellular inflammation, cell death, abnormal cell replication via blockage of DNA repair mechanisms and damage to DNA molecule (Chang et al., 2013). The results herein showed positive clastogenic effects of Dichlorvos in hematopoietic cells of *H. bidorsalis*. Muranli and Güner (2011) reported that lambda-cyhalothrin exposure induced significant micronuclei disorders in

mosquitofish (*Gambusia affinis*) after 48 h period. Further in support of the cytogenotoxic markers observed in the present study for *H. bidorsalis* exposed to sub-lethal concentrations of Dichlorvos, Amaeze et al., (2020) also reported pesticide induced MN and NA (binuclei (BN), bud shaped nuclei, lobed shaped nuclei, 8-shaped nuclei, notched nuclei and blebbed shape) in African Catfish (*Clarias gariepinus*) during 21 days of exposure.

With emerging evidence that Dichlorvos can induce immunological suppression, oxidative stress, and DNA damage in *H. bidorsalis*, it is suggested that exposure may lead to subtle but persistent physiological impairments that reduce resistance to infection, compromise reproductive success, and ultimately affect long-term survival (Díaz-Resendiz et al., 2019). Chronic immune suppression combined with oxidative and genotoxic damage has been widely reported to cause population-level consequences in fish, including reduced fitness and gradual population declines, even in the absence of high acute mortality (Amaeze et al., 2020). In aquaculture, exposure to pesticides can undermine fish health thereby increasing susceptibility to disease outbreaks and reducing growth performance (Díaz-Resendiz et al., 2019; Yang et al., 2021). When combined with other aquaculture-related stressors such as suboptimal nutrition, high stocking density, or fluctuating water quality, these physiological disturbances may result in significant economic losses and compromised animal welfare (Sharma, et al., 2020; Hoseinifar et al., 2021).

Conclusion

This study demonstrates that immune and physiological indices, including total immunoglobulin, serum lysozyme, albumin, white blood cell counts, and total protein, are significantly suppressed when *Heterobranchus bidorsalis* was exposed to Dichlorvos. Furthermore, significant cellular and nuclear abnormalities were observed by sub-lethal concentrations of Dichlorvos, indicating the pesticide's potential for genotoxicity. These results suggest that Dichlorvos presents significant sub-lethal dangers that may compromise fish cellular and genetic integrity. Fish health, population sustainability, and disease susceptibility in contaminated aquatic habitats, as well as production and biosecurity in aquaculture systems, may all be significantly impacted by such consequences. To further understand the long-term ecological and aquaculture effects of Dichlorvos pollution, more research on chronic exposure, multiple stressor interactions, and molecular pathways should be considered. In addition, the genotoxic effects of Cyclophosphamide on the *H. bidorsalis* may need to be further elucidated as some inconsistencies were noticed across various end points.

Ethical Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The methodologies were approved by the University of Ibadan Animal Care and Use Research Ethics Committee, with approval number UI-ACUREC/030-0421/27.

Funding Information

The authors received no specific funding for this work.

Author Contribution

FEO: Conceptualization, data curation, formal analysis, investigation, validation, visualization, original draft, review & editing EKA: Conceptualization, supervision, data curation, investigation, validation, visualization, review and editing AJ: Conceptualization, Supervision, review and editing TH: Data curation, analysis, Investigation, Methodology, original draft. EIA: Conceptualization, investigation, data curation, analysis, review and editing

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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